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<b>13. ABSTRACT (Maximum 200 Words)</b> The purpose of this study is to determine the roles of steroid receptor coactivators in breast cancer. The scope of the research is to assess the expression and functions of SRC-1 family members (SRC-1, GRIP1, and AIB1) in normal mammary gland and in breast tumors. We found that the expression of SRC-1 family members was regulated by ovarian hormones during mammary gland development. We also found that AIB1 protein was overexpressed in human breast cancer specimens, as compared to adjacent normal breast tissue. In addition, functions of these coactivators have been studied during the funding period. We have constructed adenovirus expressing these three coactivators. In addition, increased expression of AIB1 in human breast cancer cells stimulated the S phase entry during cell cycle progression, indicating the involvement of AIB1 in cell cycle control and cell growth of human breast cancer cells. Furthermore, overexpression of AIB1 dramatically increased the phosphorylation of Akt and Akt kinase activity in human breast cancer cells. AIB1 was also able to stimulate c-Src signaling. These studies provide important insights how AIB1 works in human breast cancer cells to promote breast cancer progression.				
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## **Introduction**

The subject of the current studies is to determine the expression and functions of steroid receptor coactivator-1 (SRC-1) family members (SRC-1, GRIP1, and AIB1) in normal mammary gland and in breast cancer. We have evaluated the expression of SRC-1 family members in human breast tumors and found that AIB1 was overexpressed in many breast tumor specimens. The scope of the research is assessing the functions of SRC1 family members during breast cancer progression. We also assess the functions of SRC-1 family members by constructing adenovirus expressing coactivators and determine the molecular actions of these coactivators in human breast cancer cells.

## **Body**

Task 1: Completed. Collection of mammary gland samples has been completed.

Task 2: Completed. We have analyzed the expression of SRC-1, GRIP1 and AIB1 in rat mammary gland using antibodies specifically against these proteins. We found that SRC-1, GRIP1, and AIB1 levels are elevated in mammary gland during pregnancy, as compared to virgin mammary gland. It is interesting to note that SRC-1 was colocalized with estrogen receptor (ER) alpha in mammary epithelium in pregnant female rats and that the expression of SRC-1 was regulated by ovarian steroid hormones. Interestingly, SRC-1 remained segregated from ER alpha in involuting mammary epithelium. GRIP1 and AIB1 were also expressed in rat mammary epithelial cells. Our data suggest that regulation of the expression of SRC-1 may be important in determining the transactivation function of ER alpha during the development of normal mammary epithelium.

Task 3: Eliminated per contract agreement.

Task 4: Completed. We have treated animals with hormones, and mammary gland samples have been collected.

Task 5: Completed. We have examined the expression of coactivators in mammary gland upon hormone treatment. Since the expression of SRC-1 family members was elevated in pregnant mammary gland, we speculated that pregnancy hormones estrogen and progesterone were able to regulate the expression of SRC-1 family members. Rats were ovariectomized to reduce the circulating estrogen and progesterone levels. One week later, estrogen and progesterone, alone or in combination, were administered. Rat mammary glands were dissected for the analysis of SRC-1 family members using western blot analysis. We found that administration of estrogen or progesterone alone decreased the expression of SRC-1. However, the expression of SRC-1 was elevated by the treatment of estrogen and progesterone. Injection of Ad-CMV-beta galactosidase reporter into mammary gland for the assessment of ER transactivation function also demonstrated that treatment with both estrogen and progesterone was able to stimulate the ER transactivation function in mammary gland. Our data suggest that increased expression of SRC-1 during pregnancy was able to enhance the ER transactivation function. A manuscript is in preparation to document these observations.

Task 6: Completed. We have completed the collection and embedding of human breast tumor specimens.

Task 7: Completed. The immunohistochemical staining of SRC-1 and AIB1 in human breast tumors has been completed. AIB1 was overexpressed in human breast tumors, after comparing 122 clinical specimens (tables 1 and 2). We are facing background problems using TIF2/GRIP1 antibody for immunohistochemical staining. We are still trying to improve this background problem.

Task 8: Completed. A manuscript is in preparation to document the staining data and correlation with clinical pathological parameters.

Task 9: Completed. We have done immunostaining of human breast cancer MCF-7 cells using anti-SRC-1 and anti-AIB1 antibodies. Nuclear staining of SRC-1 and AIB1 were observed. To a less degree, the cytoplasmic staining was observed. This is in contrast to the tumor staining we have performed. In several cases, we observed the cytoplasmic staining of SRC-1 and AIB1 in tumor cells in human breast tumors. We are not certain whether the hormonal level in patients or cells will influence the localization of SRC-1 or AIB1 in cells.

Task 10: Completed. To examine the expression of p160 coactivators in human breast cancer cell lines, we have done Western blot analysis for SRC-1, GRIP1, and AIB1 in several human breast cancer cell lines. Both ER $\alpha$ -positive (MCF-7, T47D, and ZR-75-1) and ER $\alpha$ -negative (HCC1937, MCF10A, MDA-MB-231, MDA-MB-435S, BT-20, and SKBR) cells express all three coactivators. MCF-7 cells expressed a much higher level of AIB1 among all the cell lines tested.

Task 11: Completed. We have successfully constructed the sense construct of the adenovirus expressing AIB1, GRIP1/TIF2 and SRC-1. These viruses are tagged with GFP in order to monitor the infection and expression efficiency of this virus. The control virus AdGFP has also been constructed.

Task 12: Completed. We have completed the testing for adenoviruses constructed in tissue culture cells. We have performed Western blot analysis and are able to show that AdGFP-AIB1 express functional AIB1. Preliminary data indicate that overexpression of AIB1 increased the S phase of cell cycle in T47D cells and MCF-7 cells (figure 1). Addition of PI3K/Akt inhibitor LY294002 abolished the AIB1 enhanced S phase entry (figure 2). Infection of human breast cancer cells with AIB1 increased Akt phosphorylation (figure 3) and Akt kinase activity (figure 4), suggesting the involvement of Akt signaling in mediating AIB1 regulated cell cycle progression. Furthermore, AIB1 was able to interact with c-Src in immunoprecipitation experiment (figure 5), an important adaptor protein for growth factor signaling. In transfection experiment, AIB1 and c-Src was able to synergistically activate ER transactivation function (figure 6). These data suggest that cross-talk of AIB1 signaling with growth factor signaling is playing an important role in mediating AIB1 actions in human breast cancer cells.

Task 13: Completed. Large-scale preparation of adenoviruses has been completed through CsCl banding.

Task 14: Completed. We have infused the purified adenoviruses into rat mammary gland for testing the effects of AIB1 on modulating ER signaling pathway in situ.

Task 15: Completed. We did liquid X-gal staining in mammary gland infused with Ad-ERE-beta galactosidase reporter adenovirus and adenovirus expressing coactivators. However, we did not

see much X-gal staining this time. We don't know why, and we are on the process of optimizing the experimental protocols.

### **Key Research Accomplishments**

- We have demonstrated the regulation of SRC-1 family members during the development of rat mammary gland.
- We have demonstrated that AIB1 protein was altered in many breast cancer tumors.
- We have successfully constructed adenovirus expressing AIB1, SRC-1 and GRIP1.
- We have generated rabbit antibody against AIB1.
- We have demonstrated that AIB1 over-expression could increase the S phase of the cell cycle. Addition of LY294002 was able to abolish the AIB1 stimulated increase of S phase entry.
- We found that AIB1 was able to interact with c-Src adaptor protein. Transfection of AIB1 with c-Src enhanced the ER transactivation function synergistically in human breast cancer cells.
- Infection of human breast cancer cells with adenovirus expressing AIB1 increased the phosphorylation of Akt and Akt kinase activity. LY294002 was able to abolish the AIB1 stimulated increase of S phase entry, suggesting the involvement of growth factor signaling pathways in mediating AIB1 action in human breast cancer cells.

### **Reportable Outcomes**

#### **Abstracts:**

1. Shim, W.-S., Turner, M.A., Santen, R.J., **Jeng, M.-H.** Up-regulation of SRC-1 Protein and Increase of ER in Situ Transactivation Function by pregnancy Hormones. 83<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2001. Oral Presentation.
2. Zhang, Q.-H., Chang, L.-Y., Vieth, E., Stallcup, M.R., Edwards, D.P., Cheng, L., Goulet, R.J., **and Jeng, M.-H.** Over-expression of Several Nuclear Receptor Coactivator Proteins in Human Breast Carcinoma. 83<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2001.
3. Long, X., Cai, A., Vieth, E., Quilliam, L.A., **and Jeng, M.-H.** Interaction of c-Src with Steroid Receptor Coactivators. Keystone Symposium D4: Nuclear Receptor Superfamily, 2002. Late break-through oral presentation.
4. **Jeng, M.-H.**, Zhang, Q.-H., Long, X., Goulet, R., Sledge, G., Li, L., and Quilliam L. Functions of Estrogen Receptor Coregulators in Breast Cancer. DOD Era of Hope Meeting, 2002.
5. Zhang, J., Chang, L.-Y., Long, X., Cai, A., Vieth, E., Kao, C., **and Jeng, M.-H.** Increased Expression of AIB1 Stimulated Akt Phosphorylation and Cell Cycle Progression in Human Breast Cancer Cells. 85<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2003.

Oral presentation.

## Conclusions

During this funding period, we have completed the tasks and evaluated the expression and molecular actions of SRC-1 family members. We have demonstrated that the expression of SRC-1 family members was regulated during the development of the mammary gland. In addition, AIB1 was overexpressed in human breast tumors. More importantly, AIB1 can activate c-Src and Akt signaling pathways. The interactions of AIB1 with c-Src and Akt signalings also provide important insights of the molecular actions of AIB1 in human breast cancer cells. The generation of adenoviruses expressing coactivators will provide us a valuable tool to assess the functional role of SRC-1 family members in ER transactivation function in mammary gland and in regulating breast cancer cell proliferation. Our current studies suggest that overexpression of AIB1 protein may contribute to increased S phase and subsequently increased cell proliferation in human breast epithelial cells.

## References

1. Shim, W.-S., DiRenzo, J., DeCaprio, J. A., Santen, R. J., Brown, M. J., Jeng, M.-H. Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proceedings of the National Academy of Sciences 96(1): 208-213, 1999.
2. Jeng, M.-H., Kao, C., Sivaraman, L., Krnacik, S., Chung, L. W. K., Medina, D., Conneely, O. M., and O'Malley, B. W. Reconstitution of estrogen dependent transcriptional activation of an adenoviral target gene in select regions of the rat mammary gland. Endocrinology 139(6): 2916-2925, 1998.
3. Shim, W.-S., Turner, M.A., Santen, R.J., Jeng, M.-H. Up-regulation of SRC-1 Protein and Increase of ER in Situ Transactivation Function by pregnancy Hormones. 83<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2001. Oral Presentation.
4. Zhang, Q.-H., Chang, L.-Y., Vieth, E., Stallcup, M.R., Edwards, D.P., Cheng, L., Goulet, R.J., and Jeng, M.-H. Over-expression of Several Nuclear Receptor Coactivator Proteins in Human Breast Carcinoma. 83<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2001.
5. Long, X., Cai, A., Vieth, E., Quilliam, L.A., and Jeng, M.-H. Interaction of c-Src with Steroid Receptor Coactivators. Keystone Symposium D4: Nuclear Receptor Superfamily, 2002. Late break-through oral presentation.
6. Jeng, M.-H., Zhang, Q.-H., Long, X., Goulet, R., Sledge, G., Li, L., and Quilliam L. Functions of Estrogen Receptor Coregulators in Breast Cancer. DOD Era of Hope Meeting, 2002.
7. Zhang, J., Chang, L.-Y., Long, X., Cai, A., Vieth, E., Kao, C., and Jeng, M.-H. Increased Expression of AIB1 Stimulated Akt Phosphorylation and Cell Cycle Progression in Human Breast Cancer Cells. 85<sup>rd</sup> Annual Meeting of the Endocrine Society, June, 2003. Oral presentation.

## **Appendices**

Two tables, 6 figures and four abstracts.

Table 1

**Clinicopathological factors of the breast carcinoma patients**

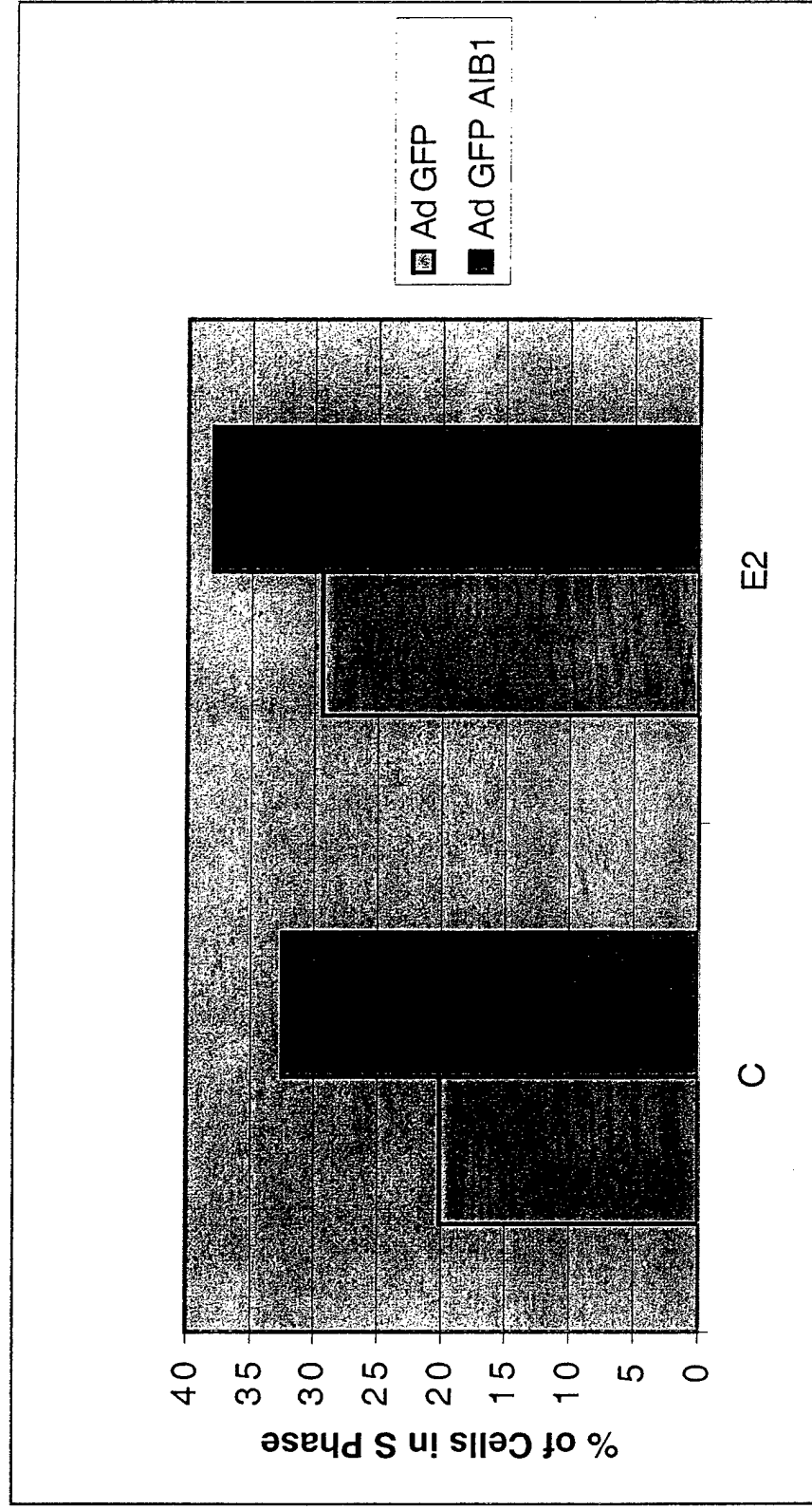
<b>Factor</b>	<b>Number</b>	<b>Percentage (%)</b>
Age		
< 50	39	31.97
>=50	83	68.03
Tumor size		
< 2cm	57	53.27
2.1-5cm	44	41.12
> 5cm	6	5.61
Lymph node		
Negative	56	53.85
Positive	48	46.15
Staging		
1	35	35.00
2	54	54.00
3	11	11.00
Grading		
Well	11	10.68
Moderate	49	47.57
Poor	43	41.75
ER status		
Negative	36	29.51
Positive	86	70.49
PR status		
Negative	71	58.20
Positive	51	41.80

Table.2

**AIB1 Immunoactivity of Normal Breast tissue and Breast Carcinoma**

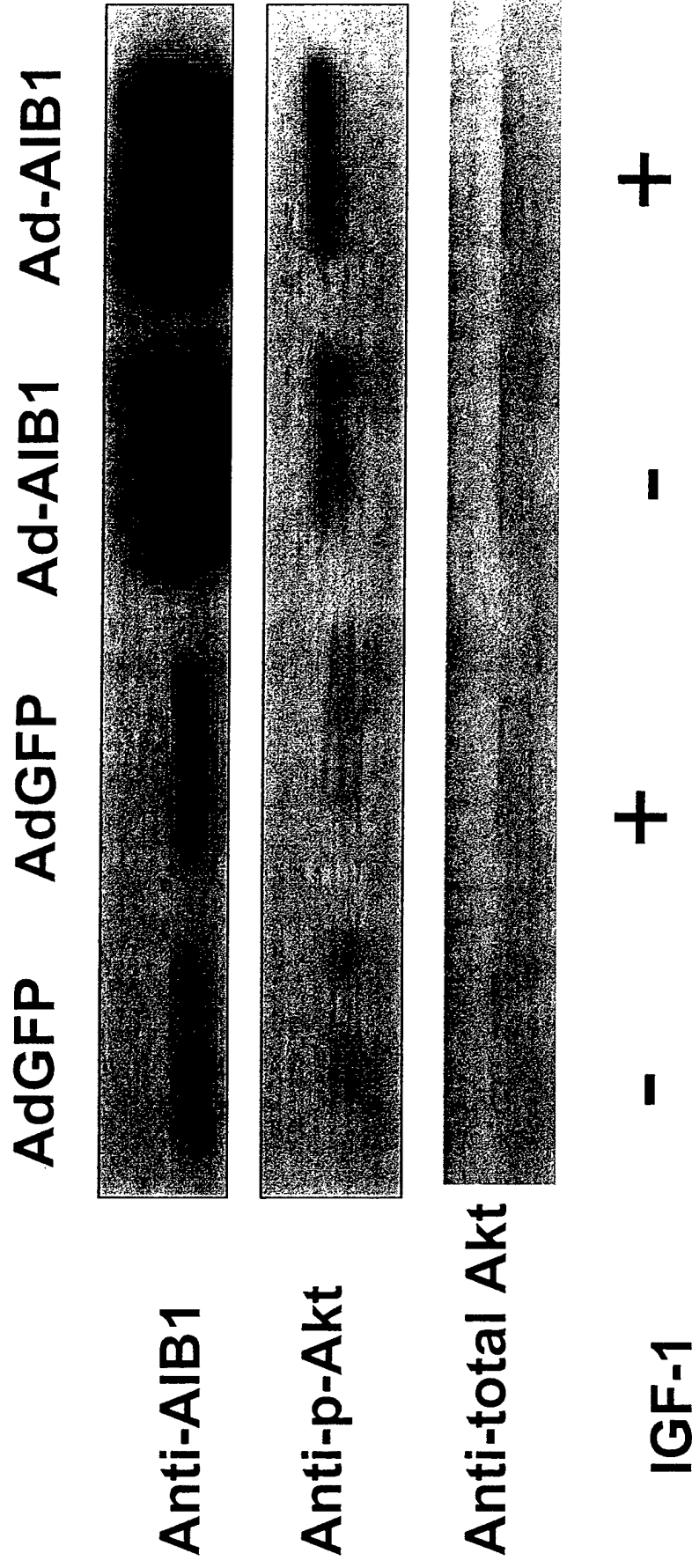
		AIB1 Staining Intensity			
	n	0	1	2	3
breast reduction tissue	9	1	6	2	0
adjacent Breast tissue	105	37	50	17	1
carcinoma	122	18	53	34	17

**Figure1 . AIB1 stimulated S phase entry  
in T47D cells**

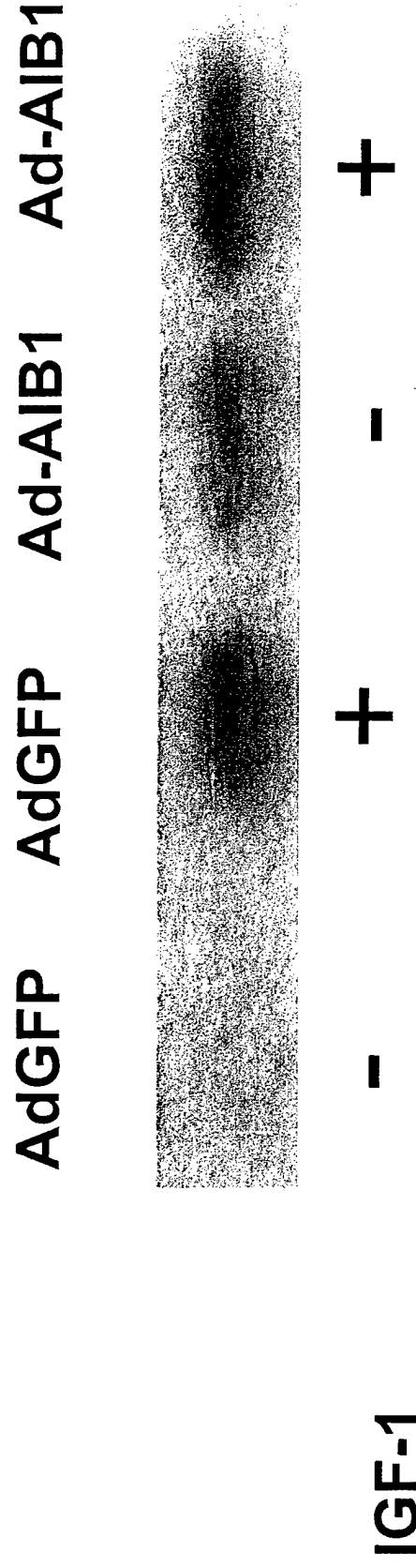




**Figure 3. Ad-AIB1 overexpression increased Akt phosphorylation in MCF-7 cells**



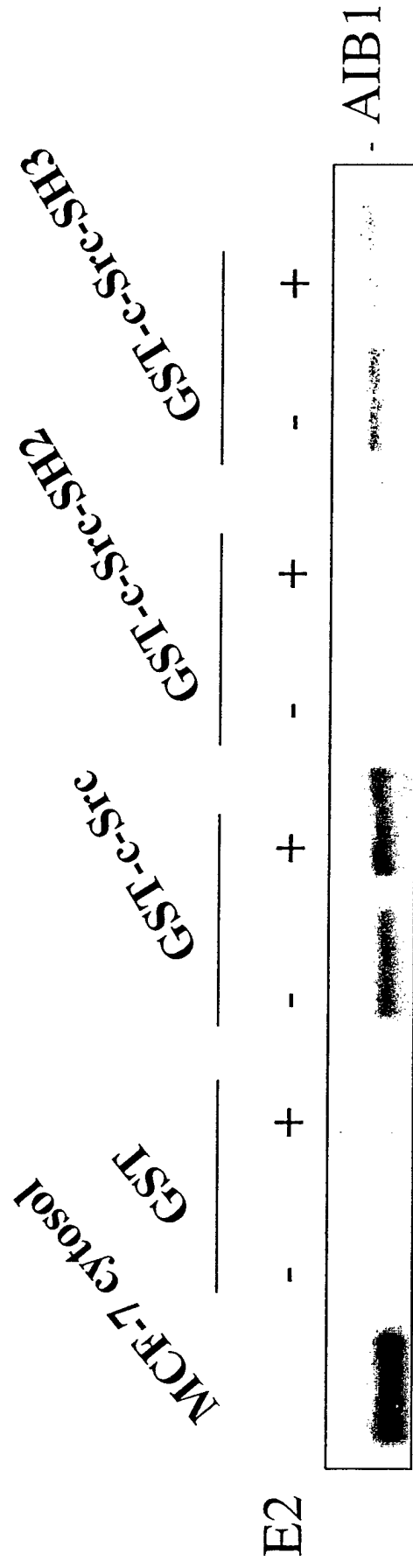
**Figure 4. Ad-AIB1 overexpression increases Akt kinase activity in MCF-7 cells**



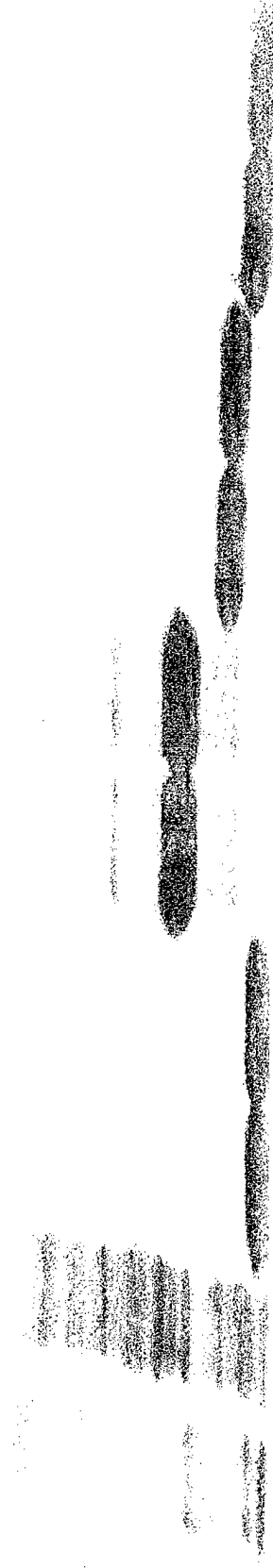
**Use histone H2B  
as the substrate in Akt kinase assay**

**Figure 5. Interaction of AIB1 with c-Src in MCF-7 cells  
in immunoprecipitation experiment**

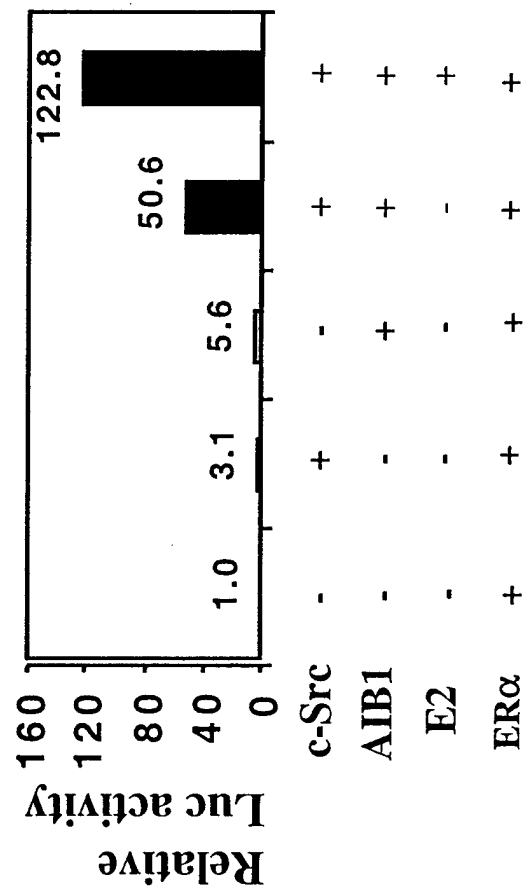
Western blot:  $\alpha$ AIB1 Ab



Ponceau S Staining



**Figure 6. Synergism of AIB1 and c-Src on ER transactivation function in transfection experiment**





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## Abstract Preview

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**UP-REGULATION OF SRC-1 PROTEIN AND INCREASE OF ER IN SITU TRANSACTIVATION FUNCTION BY PREGNANCY HORMONES.**

W.S. Shim<sup>1</sup>, M.A. Turner<sup>1</sup>, R.J. Santen<sup>1</sup> and M.H. Jeng<sup>2</sup>. <sup>1</sup>Department of Medicine, University of Virginia Health System, Charlottesville, VA, United States, <sup>2</sup>Department of Medicine, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN, United States, <sup>3</sup>Department of Medicine, University of Virginia Health System, Charlottesville, United States

Steroid Receptor Coactivator-1 (SRC-1) has been demonstrated to be a coactivator for steroid, retinoid, and thyroid hormone receptors through direct ligand-dependent interaction with these receptors. We previously demonstrated that SRC-1 was segregated from estrogen receptor  $\alpha$  (ER $\alpha$ ) and progesterone receptor (PR) in virgin female rat mammary epithelium. To further investigate the roles of SRC-1 during mammary gland development, we examined the spatial and temporal expression of SRC-1 and the effects of pregnancy hormones on SRC-1 expression using Western blot analysis, Real Time RT-PCR, and immunohistochemistry. ER in situ transactivation function was also assessed by infusing the mammary ducts with an adenoviral vector expressing  $\beta$ -galactosidase reporter under the control of an estrogen response element (Ad-ERE- $\beta$ gal). We found that SRC-1 was up-regulated and co-localized with ER $\alpha$  in mammary epithelium in pregnant female rats. Furthermore, SRC-1 remains segregated from ER $\alpha$  in mammary epithelial cells in involuting female rats. Treatment of ovariectomized rats with estrogen (E) alone was able to stimulate the Ad-ERE- $\beta$ gal reporter activity, but not the segregation status of SRC-1 from ER $\alpha$ , in rat mammary epithelium. Progesterone (P) alone was not able to influence the expression of SRC-1, the segregation status of SRC-1 from ER $\alpha$ , or the ER transactivation function. In contrast, E + P treatment (to mimic pregnancy) was sufficient to stimulate the expression of SRC-1 protein and resulted in co-localization of SRC-1 with ER $\alpha$  in mammary epithelium. More importantly, E + P treatment greatly enhanced the Ad-ERE- $\beta$ gal reporter activity in mammary gland. Our data suggest that up-regulation of SRC-1 is important in the ER transactivation function during pregnancy and that SRC-1 is involved in the full development of the mammary gland. Supported by DOD Breast Cancer Research Program DAMD17-97-1-7066 and DAMD17-99-1-9430 and RO1CA82565 (to M.-H.J.).

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### **Interaction of c-Src with Steroid Receptor Coactivators**

Xinghua Long<sup>1,3</sup>, Aijun Cai<sup>1,3</sup>, Edyta Vieth<sup>1,3</sup>, Lawrence A. Quilliam<sup>2,3</sup>,  
and Meei-Huey Jeng<sup>1,3</sup>

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Center, Indianapolis, Indiana, USA, 46202

Estrogen receptor  $\alpha$  (ER $\alpha$ ) regulates transcription of target genes by recruiting coactivator proteins, including three p160 family members, steroid receptor coactivator 1 (SRC-1), transcriptional mediators/intermediary factor 2 (TIF2/GRIP1) and amplified in breast cancer 1 (AIB1/RAC3/pCIP). Increasing evidence has suggested that cross-talk of steroid receptors with growth factor signaling components may play important roles in regulating gene expression mediated by steroid receptors. In this report, we examined the roles of steroid receptor coactivators in cross-talk of growth factor signaling with steroid receptors. We found that several adapter proteins made up of Src homology (SH) domains, including c-Src, Grb2, and PLC $\gamma$  interacted with ER $\alpha$  in GST-pulldown assays. Interestingly, these same adapter proteins could also interact with SRC-1, GRIP1, and AIB1. SH2 domain of c-Src was required for its interaction with ER $\alpha$ . However, SH3 domain was required for c-Src interaction with p160 coactivators. Interactions of c-Src with ER $\alpha$  and p160 coactivators were further confirmed by co-immunoprecipitation experiments. Protein Kinase assay indicated that c-Src phosphorylated AIB1, and the phosphorylation site was located at the C-terminal region of the AIB1 containing the multiple LXXLL motifs important for interaction with steroid receptors. This study suggests that coactivators can serve as the convergent point linking growth factor signaling to steroid receptor and that phosphorylation is likely a critical event for this type of gene regulation. Supported by NCI-RO1-CA82565, DOD and Walther Oncology Institute. MHJ is supported by DOD Breast Cancer Research Program Career Development Award.

## FUNCTIONS OF ESTROGEN RECEPTOR COREGULATORS IN BREAST CANCER

Meei-Huey Jeng, Qinghui Zhang, Xinghua Long, Robert  
Goulet, George Sledge, Liang Cheng, Lang Li, and  
Lawrence Quilliam

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Estrogen signaling components have been shown to be important in breast cancer progression. These include estrogen receptor alpha (ER) and its coregulators such as steroid receptor coactivator 1 (SRC-1) family (p160) members, SRC-1, SRC-2/TIF2/GRIP1, and SRC-3/AIB1. These ER coregulators can regulate ER transactivation function in a ligand dependent fashion. Increasing evidence suggests that the cross-talk of steroid receptors with growth factor signaling may have an important role in regulating gene expression mediated by steroid receptors. This report examines the expression of SRC-1 family members in human breast tumors and the role of ER coregulators in cross-talk between growth factors and ER signaling. We analyzed 122 cases of breast carcinoma obtained from the IU Tissue Procurement Facility, 79 cases of breast carcinoma in tissue array slides obtained from NCI, and 29 cases of breast carcinoma in commercial breast sausage slides (containing an additional 15 cases of fibroadenoma, and 15 cases of normal breast tissue). We found that AIB1 protein was overexpressed (p-value < 0.001). When normal breast epithelial MCF10A cells and breast cancer MCF-7 cells were infected with an adenovirus expressing AIB1, the S phase was increased dramatically, suggesting that AIB1 was involved in cell cycle regulation. Furthermore, several growth factor signaling adapter proteins made up of Src homology (SH) domains, including c-Src, Grb2 and PLC $\gamma$ , interacted with ER in GST-pulldown assays. Interestingly, these same adapter proteins could also interact with SRC-1, GRIP1, and AIB1. The SH2 domain of c-Src was required for interaction with ER. The SH3 domain of c-Src was required for interaction with p160 coactivators. Interactions of c-Src with ER and p160 coactivators were further confirmed by co-immunoprecipitation. Protein kinase assay indicated that c-Src phosphorylated AIB1. The phosphorylation site was located at the C-terminal region of AIB1 containing the multiple LXXLL motifs important for interaction with steroid receptors. Our data suggest that coactivators can be the convergent point linking growth factor signaling to ER and that phosphorylation is likely a critical event for this type of gene regulation in breast cancer cells.

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**Sponsor:** Meei-Huey Jeng **Member ID Number:** 162085

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**Title:** Increased Expression of AIB1 Stimulates Akt Phosphorylation and Cell Cycle Progression in Human Breast Cancer Cells

Jie Zhang <sup>1\*</sup>, Li-Yun Chang <sup>1</sup>, Xinghua Long <sup>1</sup>, Aijun Cai <sup>1</sup>, Edyta Vieth <sup>1</sup>, Chinghai Kao <sup>1</sup> and Meei-Huey Jeng <sup>1</sup>. <sup>1</sup> Departments of Medicine and Urology, and the Walther Oncology Center, Indiana University School of Medicine, and the Walther Cancer Institute, indianapolis, indiana, 46202 .

Overexpression of AIB1 (amplified in breast cancer1) has been demonstrated in human breast cancer cells. AIB1 can act as a coactivator for steroid receptors and can potentiate the transactivation function of estrogen receptor (ER) in human breast cancer cells. However, the precise role of AIB1 in breast cancer progression remains to be determined. To elucidate the contribution of AIB1 in regulating breast cancer cell proliferation, we constructed adenovirus overexpressing AIB1 and green fluorescent protein (GFP), Ad-GFP-AIB1. The control adenovirus Ad-GFP, expressing only the GFP, was also generated. Transduction of breast cancer cells with Ad-GFP-AIB1 dramatically increased the expression of AIB1, as compared to cells transduced with Ad-GFP control virus. To determine whether AIB1 overexpression was able to modulate cell cycle progression, we transduced breast cancer cells with Ad-GFP-AIB1 virus and observed increased S phase entry when AIB1 was overexpressed. Inhibition of AIB1 expression by siRNA technique abolished the increased S phase entry induced by estrogen. Furthermore, we found that increased AIB1 expression activated and phosphorylated the serine/threonine kinase Akt, an important signaling component for growth factor and cytokine receptors. The activation of Akt phosphorylation by AIB1 could be further enhanced by the addition of insulin-like growth factor-I. Transfection of constitutively active Akt in breast cancer cells was able to enhance the AIB1-stimulated ERE reporter activity, suggesting that activation of Akt can feed back to regulate AIB1-stimulated ER transactivation function. Interestingly, we also found that Akt was able to phosphorylate AIB1, indicating that AIB1 is a substrate for and a downstream effector of Akt. Our data strongly suggest

the notions that overexpression of AIB1 can regulate breast cancer cell proliferation and that Akt phosphorylation is, at least in part, responsible for the AIB1 action. Moreover, phosphorylation of AIB1 by Akt can subsequently activate Akt through a feedback mechanism in human breast cancer cells.

**References:**

**Financial Support:** Supported by NIH, DOD, and Walther Cancer Institute to MHJ.

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